

## Effects of the use of feeds containing phytase enzyme from different protein sources on nitrogen and phosphorus discharge of rainbow trout (*Oncorhynchus mykiss*) juveniles

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### Abstract

In this study, the effects of diets (D<sub>1-3</sub>) including fish meal (FM), hazelnut meal (HM), soybean meal (SM), and phytase enzyme [- (0), +(1000 FTU)] in varying rates (D<sub>1-</sub>, D<sub>1+</sub>; D<sub>2-</sub>, D<sub>2+</sub>; D<sub>3-</sub>, D<sub>3+</sub>) were examined on nitrogen and phosphorus discharge based on the nutrition of rainbow trout (*Oncorhynchus mykiss*) juveniles. The study was conducted in tanks with 3 replicates for each group. In this trial, it was found that the differences among total-particle nitrogen, particle phosphorus, and total solid waste values released from the rainbow trout groups were insignificant; however, the differences between the values of total-dissolved phosphorus and dissolved nitrogen released were found significant ( $p < 0.05$ ). The interactions among the factors were determined as insignificant ( $p > 0.05$ ). Moreover, although there were increases in the total and dissolved phosphorus and dissolved nitrogen discharge in all groups fed with diets including phytase, the particle phosphorus discharge decreased ( $p < 0.05$ ). Based on the decrease in the amounts of hazelnut meal and soybean meal used in the diets, it was determined that there was a decrease in the amount of dissolved phosphorus released to the environment; besides, it was determined that it led to an increase in the amount of released dissolved phosphorus in all groups fed with diets containing phytase enzyme ( $p < 0.05$ ).

**Keywords:** Diets protein, Waste, Dissolve, Enzyme, Fish

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## Introduction

Along with the development of aquaculture, demand for these products has also increased on a global scale. As a result of this, organic and inorganic waste loads have increased causing contamination and serious apprehensions (Frankic and Hershner, 2003; Subasinghe *et al.*, 2009; Herath and Satoh, 2015). It was reported that potential contaminants are dissolved-particle nitrogen and phosphorus (Herath and Satoh, 2015). The fish receive nitrogen and phosphorus from the feeds and they use a small portion of them in their biological cycle; however, it is reported that the rest is released into the ecosystem in particle forms. It was stated that metabolic waste and unconsumed feed sources were the most contaminative nutrient inputs to the coastal and marine environments from the fish farms. According to the asserted hypothesis, it was reported that 18.5% of the nitrogen and 14.3 % of the phosphorus in the feeds were consumed by the fish; 81.5% of the nitrogen and 85.7% of the phosphorus were released into the environment for each ton of fish production (Islam, 2005).

Reduction/removal of nitrogen and particularly ammonia is vital for the ideal quality of water in aquaculture, as well as for the well-being and health of the fish. It is because the ammonia released based on catabolism of feed protein may cause stress in the fish, preventing their growth; therefore it is reported as a limiting factor and a problem (Crab *et al.*, 2007; Dalsgaard and Pedersen, 2011).

In recent years, important legal regulations were made as a solution to environmental problems caused by these reasons (Subasinghe *et al.*, 2009). In this context, in many countries good management implementations have been obligatory such as selection of production site and the sources, enhancement of the feed and feeding programs, prevention of fish escape, control of predators, removal of the dead fish, control of the diseases, inspection of the use of chemicals and drugs, removal of the solid waste, and waste treatment (Cho and Bureau, 2001; Tacon and Forster, 2003; Tucker *et al.*, 2008). In this regard, a good example can be inland waters of Europe that are quite clearer compared to 25 years before (EEA, 2015). Big amounts of droppings were released daily from intense aquaculture systems, and the level of the organic waste discharged from these systems varied based on the quality, amount, digestibility, and usage proportions (Dalsgaard and Pedersen, 2011). Demir (2011) stated that the 30-70% of the total cost in aquaculture was the cost of feeds. Based on the growth of the aquaculture sector, the fish feed industry increased the unit cost of the feeds since they started using imported fish meal. It was stated that, instead of fish meal, the sector is inclined towards using various herbal seeds and the legumes in feed production as protein sources (Ruohonen, *et al.*, 2007; Granada *et al.*, 2016). It was reported that the antitrypsin factor found in some legumes is largely removed by heat application, which is then used in feeding animals (Uysal and Bekcam,

2006; Harlioğlu, 2011). Important findings were reached on the usage of hazelnut meal instead of fish meal in the diets (Emre *et al.*, 2008a, Emre *et al.*, 2008b; Sevgili *et al.*, 2009a, Sevgili *et al.*, 2009b). It was mentioned that, soybean protein can be used partially instead of fish meal in feeds to decrease environmental pollution based on feeds, without compromising the live weight of fish and without benefiting from the feeds in rainbow trout production (Vielma *et al.*, 2000). Phosphorus was reported to be an important limiting nutrient for life in freshwater systems, and it was also reported that, through various human activities (eg. industrial and municipal sewage treatments, aquaculture), the excessive amount of phosphorus entering the lake ecosystem through rivers caused eutrophication and algal blooms (Jia *et al.*, 2015). It was reported that nitrogen loss in aquaculture in cages varied between 72% and 79% and that approximately 82% of the waste was in the dissolved form. In aquaculture, 65-90% of total nitrogen loss is released via gills and metabolic droppings. Waste nitrogen is in the form of ionized and non-ionized ammonia. The risk of eutrophication is increasing with the involvement of nitrogen in the receiving environment. The amount of nitrogenous and phosphorus wastes entering the receiving environment depends on the protein level and digestibility of the feed. Phosphorus requirements of young salmon fish were reported to be 0.25-0.4 g MJ<sup>-1</sup> digestible energy (Vielma *et al.*, 1998). In seeds, phosphorus is involved in the structure

of phytic acid; and it is also reported that the utilization of minerals along with the digestion of protein and starch is also reduced due to the ability of phytic acid molecules to chelate. It is also reported to inhibit the activity of the enzyme  $\alpha$ -amylase and trypsin by binding calcium (Thompson *et al.*, 1987). Phytase enzymes (myo-inositol-hexakisphosphate-3-phosphohydrolase) are separated from each other by 3-phytase (in microorganisms) and 6-phytase (plants) according to binding of inositol, in its structure, to the carbon atom. Because phosphorus in legumes is phytate, its use and bioavailability by fish and all monogastric animals is very low. It was reported that when phosphorus is inadequately utilized in the feeding process it causes phosphorus insufficiency; in this case, it leads to an increase in the cost of feed production as it requires the addition of inorganic phosphorus to mixed feeds. However, it was reported that the use of feed supplemented with phytase enzyme significantly increased the value of fish and the profit index (Orisasona *et al.*, 2017). The use of microbial enzymes in biotechnological applications is also stated to be promising.

The addition of phytase enzyme to feeds increases digestibility of feed minerals, protein-amino acids, and starch; it also increases the conversion rates from feed, thus reducing fecal contamination caused by animal waste (Cao *et al.*, 2007). The use of the 2000 FTU phytase enzyme in the diet was reported to significantly reduce phosphorus discharge (Biswas *et al.*,

2007). It was suggested that the addition of phytase enzyme to the diet, which contained a high amount of herbal protein and a low level of phosphorus, would be advantageous for fish and the environment. However, it was noted that fungal phytase should not be added to diets, which meet the requirements of the fish with their phosphorus levels, but would lead to a significant increase in the amount of dissolved and suspended phosphorus waste (Dalsgaard *et al.*, 2009). It was reported that the increase in the amount of herbal protein in the diets reduced the utilization of the phosphorus in the fish diet (Cheng *et al.*, 2010). For an estimated production of 1 ton of tilapia (*O. niloticus*), 14.8 kg phosphorus was discharged to the receiving environment (David *et al.*, 2015). In offshore cage systems, the amounts of total, dissolved, and particle nitrogen discharged into the receiving environment during the production of one ton of fish were 44.4 kg, 37.5 kg, 6.9 kg, respectively; and the amounts of total, dissolved, and particle phosphorus values were 5.7 kg, 2.5 kg and 3.2 kg, respectively (Maar *et al.*, 2018). It was reported that phosphorus discharge to the receiving environment was 9.38 kg in the production of one ton of trout, and phosphorus discharge was 8.09 kg in utilization of an average 1 ton of feed (Pulatsü *et al.*, 2004). In the production of one ton of fish, 180 kg of solid waste, 13 kg of phosphorus and 105.4 kg of nitrogen were discharged to the receiving environment (Hasan, 2001). In trout production, the amounts of droppings released, total feed-based

substances, particles, and dissolved nitrogen, and phosphorus discharge were reported as 236, 12.8, 5.3, 41.3 and 3.4 kg ton<sup>-1</sup> fish, respectively in 2003, and these values were reported as, respectively, 220, 12.3, 5.3, 38 and 3.4 kg ton<sup>-1</sup> fish, respectively in 2004. It was estimated that more than 60% of phosphorus waste in cages was in particle form, which was the same in 2003 and 2004, but more than 65% of total nitrogen was in ammonia form. The amount of dissolved and particle phosphorus and ammonia did not differ according to the locations of the cages, and that it was effectively removed via nitrification by the biota (Azevedo *et al.*, 2011). It was reported that, the feed loss varied between 8.56-52.2%, and the total particle discharge amount varied between 59.17-134.71 kg day<sup>-1</sup> in the production of sea bream and sea bass in the offshore cages (Ballester-Moltó *et al.*, 2017). Aşır and Pulatsu (2008) found that, for the production of one ton of rainbow trout in a dam lake, when using pellet feeds, 54.00-62.92 kg nitrogen and 10.66-12.17 kg phosphorus were discharged to the receiving environment; and when extruder feeds were used, 33.47-25.97 kg nitrogen and 7.32-7.96 kg phosphorus were discharged. The amounts of nitrogen and phosphorus released into the lake were estimated by these enterprises to be 44.00-45.56 kg and 8.38-8.82 kg, respectively, using a ton of pellet feed; and these values were estimated as 20.66-26.77 kg and 5.85-6.34 kg respectively, using extruder feeds. It was reported that, for the production of one ton of fish in cages

with feeds including 7.2 % phosphorus and 0.9 % nitrogen, the amounts of dissolved nitrogen and phosphorus released to the receiving environment were 61 kg and 2.2 kg, respectively. Additionally, there were 17 kg particle nitrogen and 7.3 kg particle phosphorus. Moreover, the conversion rate was reported as 1.5 (Ackefors and Enell, 1990). It was reported that the feed conversion rate of salmon, which were fed with feeds including 7.0% nitrogen and 1.3% phosphorus, was 1.1; its body dry weight was 10% N and 3.2% P; and nitrogen and phosphorus nutrient loads of one ton of feed were 47.7 kg and 5.7 kg, respectively (Boyd and Querioz, 2001). In the production of one ton rainbow trout in tanks, the feed conversion rate was estimated as 1.83, total nitrogen discharge as 124.2 kg and total phosphorus as 25.6 kg. They found 2.58% nitrogen and 0.40% phosphorus in the meat of the produced fish (Foy and Rosell, 1991). It was stated that by changing the contents of the feed used for aquaculture, the discharge load of nitrogen and phosphorus released from the facilities of enterprises can be reduced. As an example, in Sweden, feeds used for trout feeding in the early 1990s contained 1.2-1.4% phosphorus, and this rate was reduced to 0.8% with the development of quality feeds in the later period. It was reported that in the production of 1 ton of trout 29 kg of phosphorus was discharged into the receiving environment until 1985; as a result of these developments, this value was decreased by 15 kg in recent years (Midlen and Redding, 1998). The

addition of phytase enzyme to the rainbow trout feed increased utilization of the phosphorus concentrate of soybean protein in the feed and reduced the phosphorus load by 58% compared to the group, which was fed with a phytase-free feed. Moreover, it was reported that cholecalciferol added to the feed in moderate levels reduced phosphorus release (Vielma *et al.*, 1998). Bureau and Cho (1999) stated that phosphorus waste was particularly in the dissolved form in fish farming activities and that effective phosphorus adsorption was decreased with increasing phosphorus intake. Hernandez *et al.* (2004) reported that phosphorus retained in the body of the rainbow trout, which were fed with feed containing 15-20% less fish meal and 0.8-0.9% less phosphorus compared to the control group, was higher (56% and 69%, respectively) than that of the group, which was fed with feed containing higher phosphorous and nitrogen contents; however, the retainment rate of nitrogen was similar in all groups. They found that the proportion of phosphorus and nitrogen retained by the large fish was lower than that of the small fish. The amount of phosphorus load released to the water was determined as 5.9 kg ton<sup>-1</sup> in the group fed with the trial feed, whereas it was 12.8 kg ton<sup>-1</sup> in the control group.

In this study, trial diets were prepared containing different amounts of hazelnut meal, soybean meal, fish meal, and phytase enzyme. The effects were examined on the total, dissolved, and particle nitrogen and phosphorus

discharged to the receiving environment by rainbow trout (*Oncorhynchus mykiss*) groups, which were fed with these diets. It was aimed to reduce the released amounts of nitrogen, particularly phosphorus, in order to provide more economical, environmental, and sustainable aquaculture.

## Materials and methods

### Experimental conditions and the diets

In this study, 750 rainbow trout (*O. mykiss*) offspring (average live weight  $27.82 \pm 0.04$  g) obtained from a private company were used at the Kepez Trout Unit of the Mediterranean Fisheries Research, Development and Training Institute. After the fish were kept in

quarantine for 15 days, the trial period used in the experiment was planned to be 75 days. The raw materials used in the diets (Table 1) were obtained from Kağsan Blacksea Food and Agriculture Industry Inc. In the trial, six diets were used in feeding the offspring of the rainbow trout; the diets were prepared with equal crude protein values (% 42 HP) as not-including phytase enzyme “0 FTU” (-) and including phytase enzyme “1000 FTU” (+), with fish meal protein (FMP), hazelnut meal protein (HMP), and soybean meal protein (SMP) in different ratios ( $D_{1,-,+} = \%30$  FMP +  $\%35$  HKMP +  $\%35$  SMP;  $D_{2,-,+} = \%40$  FMP +  $\%30$  HKMP +  $\%30$  SMP;  $D_{3,-,+} = \%50$  FMP +  $\%25$  HKMP +  $\%25$  SMP).

**Table 1: Raw material ratios and contents distribution of the trial diets (%)\*.**

Raw materials	% CP	Diets					
		D <sub>1</sub> -	D <sub>1</sub> +	D <sub>2</sub> -	D <sub>2</sub> +	D <sub>3</sub> -	D <sub>3</sub> +
Phytase (FTU)	-	0	1000	0	1000	0	1000
Fish Meal	70	18	18	24	24	30	30
Soybean Meal	49	28.8	28.8	24.8	24.8	20.4	20.4
Hazelnut Meal	45	33	33	27.8	27.8	23	23
Corn gluten	60	2.4	2.4	1.7	1.7	1	1
Wheat flour	13	1.64	1.64	2.99	2.99	5.49	5.49
Corn flour	9	0	0	3.85	3.85	7.05	7.05
Mixed vitamin	-	0.5	0.5	0.5	0.5	0.5	0.5
Mixed mineral	-	0.11	0.11	0.11	0.11	0.11	0.11
Fish oil	-	14.4	14.4	13.1	13.1	11.3	11.3
Carboxymethyl Cellulose	-	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	-	0.15	0.15	0.15	0.15	0.15	0.15
Chromic oxide	-	0.5	0.5	0.5	0.5	0.5	0.5
Unit diet		100	100	100	100	100	100

\* Diet ‘D’;  $D_{1,-,+} = \%30$  FMP +  $\%35$  HMP +  $\%35$  SMP;  $D_{2,-,+} = \%40$  FMP +  $\%30$  HMP +  $\%30$  SMP;  $D_{3,-,+} = \%50$  FMP +  $\%25$  HMP +  $\%25$  SMP; Phytase ‘F’, 0 FTU ‘-’; 1000 FTU ‘+’. CP= Crude protein

The study was conducted in 18 trial tanks (400 L) in 6 different groups with 3 replicates, each stocked randomly with 25 fish. The fish were fed in the morning and evening at a rate of approximately 2% of the daily body weight. Each tank was supplied with 12

$L \text{ min}^{-1}$  of water. In the trial, the water temperature was  $17.5 \pm 0.52$  °C, the dissolved oxygen content was  $9.2 \pm 0.55$  mg  $L^{-1}$ , and the pH was  $8.0 \pm 0.08$ . The prepared trial diets were kept at +4 °C until they were used.

*Analyses and measurements*

In this study, the nitrogen and phosphorus amounts released to the environment by the rainbow trout groups which were fed with trial diets were measured. Moreover, feed conversion rates were periodically calculated, determining the feed

requirements. Dry matter, crude protein, crude lipid, crude ash (AOAC, 2000), (Table 2) and chromic oxide levels in the droppings were determined using atomic absorption (Perkin Elmer AAS800) and spectrophotometer (Hach Lange BR6000) (Furukawa and Tsukahara, 1966).

**Table 2: The proximate analysis of trial diets (%).**

Diets	Nutrient distributions of dry matter of the diets				
	Moisture	DM	CA	CL	CP
D <sub>1</sub> -	6.39	93.61	8.53	17.64	42.64
D <sub>1</sub> +	6.61	93.39	8.71	21.09	42.92
D <sub>2</sub> -	6.32	93.68	8.74	14.32	42.54
D <sub>2</sub> +	6.22	93.78	8.81	18.79	42.44
D <sub>3</sub> -	5.93	94.07	8.93	15.04	42.02
D <sub>3</sub> +	6.55	93.45	9.13	18.72	42.86

Diets 'D'; Dry Matter 'DM'; Crude Ash 'CA'; Crude Lipid 'CL'; Crude Protein 'CP'

*Phosphorus analyses and calculations*

Approximately 250 mg of sample was weighed in the microwave burning chambers and 10 mL of concentrated HNO<sub>3</sub> was added. Once the lid was tightly closed, it was placed on the Berghof brand microwave (MWS-2) burner and subjected to wet decomposition under the appropriate program. After the decomposition transaction the extracted sample was cooled to the room temperature and 1-2 drops of phenolphthalein was dropped on them. Subsequently, it was neutralized using 10 N NaOH until a pink-orange color was formed. Then, 50% HNO<sub>3</sub> was added dropwise until it turned into light yellow or colorless. Samples were made up to 50 mL with distilled water. Phosphorus levels of the samples were determined using the vanadate method and

spectrophotometer (James, 1999). The released nutrients (Vielma *et al.*, 2002) were calculated according to Cho and Bureau (2001) as solid N waste, dissolved N waste, solid P waste, and dissolved P waste (g kg<sup>-1</sup> fish).

*Statistical analysis*

Normality of the data was verified by Shapiro-Wilk W Trial, and their homogeneity was verified via Bartlett trial. All of the percentage values were evaluated after arcsin transformation, while the differences among the averages of the trial groups were trialed via one way variance analysis (ANOVA). Tukey multiple comparisons trial was used in determining the differences. Whether phytase addition was effective on the feeds was trialed via the two-way ANOVA. The analyses were conducted

via JMP 8.0 Statistical Package Program (SAS Institute and Inc., 2008), and the results were given as the standard error of the mean (mean±SEM).

## Results

The total solid waste, total, particulate, and dissolved nitrogen (N) and phosphorus (P) released were calculated by taking into account the digestibility rates of the diets depending on the mass balance approach and the trial factors and levels (Tables 1,2,3,4, Figs. 1,2,3). It was determined that the digestibility rates of the diet proteins by the trial group fish varied between 78.11% and 84.37%; it was also detected that the effects of the "diet protein source" and "phytase enzyme" factor levels, and the interactions between the "diet protein

source ×phytase enzyme" factors were similar ( $p>0.05$ ). However, the relative digestibility of diet protein of the groups fed at rates of D<sub>1+</sub>, D<sub>2+</sub>, D<sub>3+</sub> was significantly higher compared to the groups fed at the rates of D<sub>1-</sub>, D<sub>2-</sub>, D<sub>3-</sub>, which did not include phytase enzyme (Table 4). The effects of "diet protein source" and "phytase enzyme" factor levels, and the interaction between the "diet protein source×phytase enzyme" factors on the total amount of solid waste discharged from the trial groups were determined to be similar ( $p>0.05$ ). The lowest total solid waste was found in the D<sub>3</sub> + group at 443.3 kg ton<sup>-1</sup> fish, while the highest total solid waste was identified in the D<sub>2</sub> - group at 568.4 kg ton<sup>-1</sup> fish (Fig. 1).

**Table 3: The amounts of solid waste, total nitrogen, total phosphorus, particle nitrogen, particle phosphorus, dissolved nitrogen, and dissolved phosphorus released to the receiving environment from the groups fed with the trial diets (kg ton<sup>-1</sup> fish).**

Diets <sup>a</sup>	Total N discharge	Total P discharge	Particle N discharge	Dissolved N discharge	Particle P discharge	Dissolved P discharge	Total solid waste
D <sub>1-</sub>	57.88±1.65	12.23±0.75 <sup>c</sup>	13.99±1.34	43.88±1.62 <sup>ab</sup>	6.43±0.61 <sup>abc</sup>	5.80±0.20 <sup>cd</sup>	443.8±49.4
D <sub>1+</sub>	65.41±5.02	15.79±0.71 <sup>a</sup>	14.08±1.44	51.33±3.88 <sup>a</sup>	5.52±0.47 <sup>b</sup>	10.27±0.25 <sup>a</sup>	462.5±60.6
D <sub>2-</sub>	63.92±0.68	12.84±0.34 <sup>bc</sup>	17.89±1.93	46.02±1.63 <sup>ab</sup>	8.45±1.28 <sup>a</sup>	4.40±1.30 <sup>cd</sup>	568.4±67.0
D <sub>2+</sub>	62.19±1.91	14.95±0.42 <sup>ab</sup>	16.27±0.35	45.93±1.60 <sup>ab</sup>	5.42±0.18 <sup>c</sup>	9.53±0.27 <sup>ab</sup>	510.9±6.74
D <sub>3-</sub>	55.61±1.03	11.64±0.28 <sup>c</sup>	17.51±1.93	38.10±2.89 <sup>b</sup>	7.76±0.64 <sup>ab</sup>	3.88±0.57 <sup>d</sup>	550.5±62.1
D <sub>3+</sub>	58.47±1.31	14.06±0.39 <sup>abc</sup>	14.57±1.03	43.90±1.45 <sup>ab</sup>	6.78±0.60 <sup>ab</sup>	7.28±0.23 <sup>bc</sup>	443.3±36.7
Two Way ANOVA							
DPS	0.067	0.082	0.144	0.040	0.209	0.006	0.280
F	0.168	0.001	0.230	0.042	0.016	0.001	0.268
DPS×F	0.200	0.356	0.590	0.281	0.275	0.384	0.488

<sup>a</sup> The groups denoted with different letters on the same column are different from each other ( $p<0.05$ ). Diets 'D<sub>1-3</sub>'; Dietary protein source 'DPS'; Phytase 'F', 0 FTU '-', 1000 FTU '+', Nitrogen 'N'; Phosphorus 'P', The interactions between the 'diet protein source×phytase enzyme' factors 'DPS×F'.



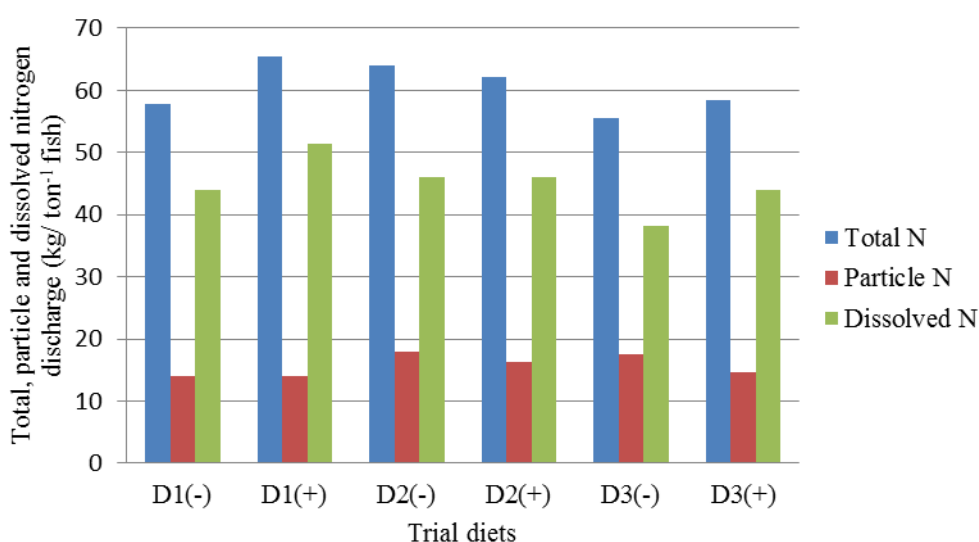
The correlation between total nitrogen and phosphorus discharge and the digestibility rate of the diet protein (Table 4, Fig. 3) was found to be negatively very weak in all groups except in the D<sub>3</sub>- group. The correlation between the total nitrogen discharge with the digestibility of the diet protein ratio was found between -0.67 and +0.88. Similarly, the correlation for total phosphorus was found to be -0.1

and -1. Moreover, this is also understood from the values of the coefficient of determination ( $R^2$ ). According to this, it can be mentioned that there are weak relations between digestibility of the diet protein and the total discharge amounts from the groups, such as 40% with the nitrogen discharge amount and 6% with the phosphorus discharge amount.

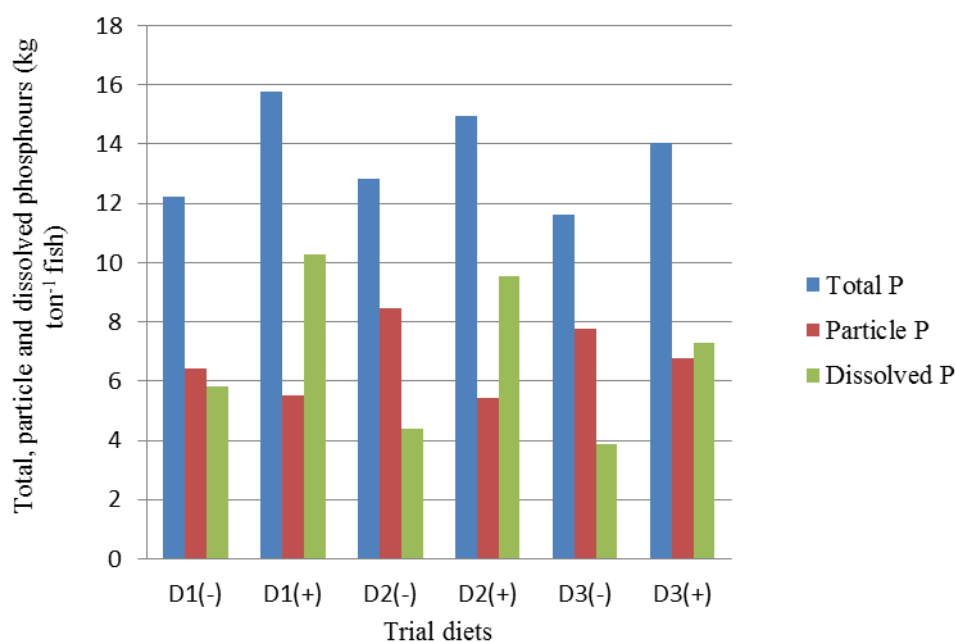
**Table 4: The correlation between the protein digestibility rate (PDR) of the diet and total nitrogen (N) and phosphorus (P) discharged.**

Diets	Protein Digestibility rate	Total N discharge (kg ton <sup>-1</sup> )	PDR-N correlation	Total P discharge (kg ton <sup>-1</sup> )	PDR-P correlation
D <sub>1</sub> -	83.07±1.43	57.88±1.65	-0.29	12.23±0.75 <sup>c</sup>	-0.69
D <sub>1</sub> +	84.37±1.12	65.41±5.02	-0.67	15.79±0.71 <sup>a</sup>	-0.85
D <sub>2</sub> -	79.70±2.12	63.92±0.68	-0.45	12.84±0.34 <sup>bc</sup>	-0.16
D <sub>2</sub> +	81.47±0.16	62.19±1.91	-0.002	14.95±0.42 <sup>ab</sup>	-0.2
D <sub>3</sub> -	78.11±2.61	55.61±1.03	0.88	11.64±0.28 <sup>c</sup>	-0.92
D <sub>3</sub> +	82.40±1.19	58.47±1.31	-0.03	14.06±0.39 <sup>abc</sup>	-1
Two Way ANOVA					
DPS	0.106	0.067		0.082	
F	0.091	0.168		0.001	
DPS×F	0.629	0.200		0.356	

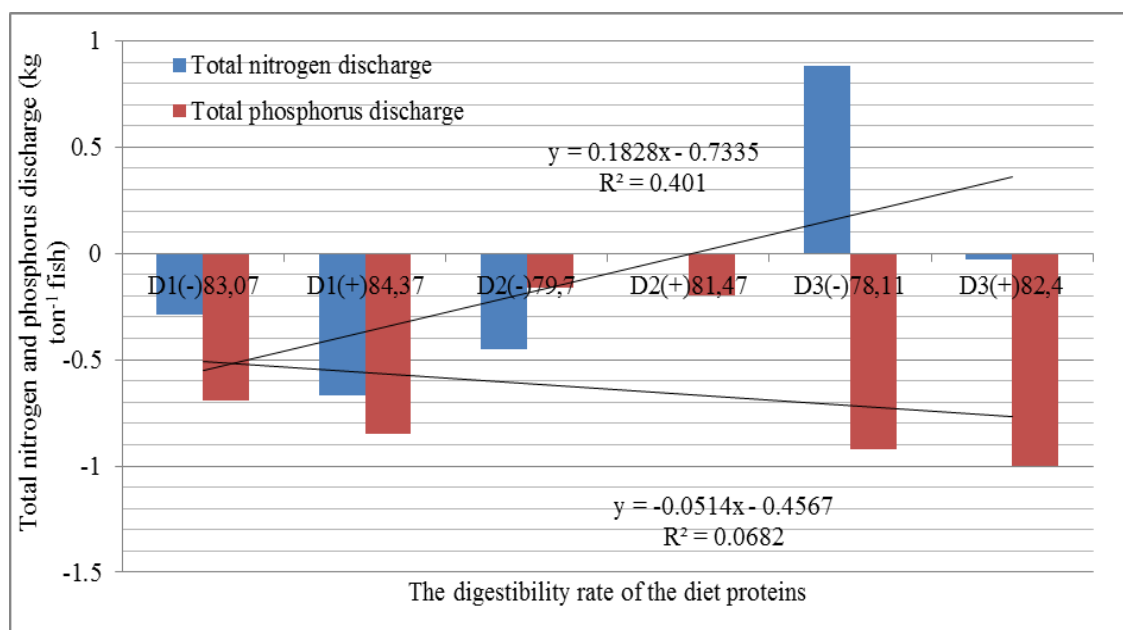
<sup>a</sup> The groups denoted with different letters on the same column are different from each other ( $p < 0.05$ ). Diets 'D<sub>1-3</sub>'; Dietary protein source 'DPS'; Phytase 'F', 0 FTU '-', 1000 FTU '+', Nitrogen 'N'; Phosphorus 'P', The interactions between the 'diet protein source×phytase enzyme' factors 'DPS×F'; Protein digestibility rate 'PDR';



**Figure 1: The total, particle, and dissolved nitrogen discharge amounts released to the receiving environment from the groups fed with the trial diets.**



**Figure 2:** The total, particle, and dissolved phosphorus discharge amounts released to the receiving environment from the groups fed with the trial diets.



**Figure 3:** The correlation between the digestibility rate of the diet proteins and the amounts of total discharged nitrogen and phosphorus.

It was determined that the effects of the "diet protein source" and "phytase enzyme" factor levels and the interactions between the "diet protein source×phytase enzyme" factors on the total discharge amount of the nitrogen released from the trial groups were insignificant ( $p>0.05$ ). The highest total

nitrogen discharge was recorded in the D<sub>1</sub>+group as 65.41 kg ton<sup>-1</sup> fish and the lowest was detected in the D<sub>3</sub>-group as 55.61 kg ton<sup>-1</sup> fish (Fig.1).

It was determined that the effects of the "diet protein source" and "phytase enzyme" factor levels and the interactions between the "diet protein

source×phytase enzyme" factors on the discharge amount of the particle nitrogen released from the trial groups were similar ( $p>0.05$ ). The lowest particle nitrogen discharge was recorded in the D<sub>1</sub> - group as  $13.99\pm1.34$  kg ton<sup>-1</sup> fish and the highest particle nitrogen discharge was detected in the D<sub>2</sub>- group as 17.89 kg ton<sup>-1</sup> fish (Fig. 1).

It was determined that the effects of the "diet protein source" and "phytase enzyme" factor levels on the discharge amount of the dissolved nitrogen released from the trial groups were significant ( $p<0.05$ ); however, the effects of the interactions between the "diet protein source×phytase enzyme" factors were insignificant ( $P>0.05$ ). The lowest dissolved nitrogen discharge was recorded in the D<sub>3</sub> - group as 38.10 kg ton<sup>-1</sup> fish and the highest was detected in the D<sub>1</sub>+ group as 51.33 kg ton<sup>-1</sup> fish (Fig. 1).

It was determined that the effects of the "diet protein source" factor level and the interactions between the "diet protein source×phytase enzyme" factors on the total amount of the phosphours released from the trial groups were insignificant ( $p>0.05$ ). However, the effects of the "phytase enzyme" factor levels on the total amount of the phosphours released from the trial groups were determined as significant ( $p<0.05$ ). The highest total phosphorus released from the trial groups was recorded in the D<sub>1</sub>+group as 15.79 kg ton<sup>-1</sup> fish and the lowest was detected in the D<sub>3</sub>-group as 11.64 kg ton<sup>-1</sup> fish (Table 3, Fig. 2). It was determined that the effects of the "diet protein source"

factor and the interactions between the "diet protein source×phytase enzyme" factors were insignificant ( $p>0.05$ ) on the discharge amount of the particle phosphorus released from the trial groups; while the effect of "phytase enzyme" factor on the discharge amount of the particle phosphorus released from the trial groups was detected as significant ( $p<0.05$ ). The highest total phosphorus released from the trial groups was recorded in the D<sub>2</sub>-group as 8.45 kg ton<sup>-1</sup> fish and the lowest was detected in the D<sub>2</sub>+ group as 5.42 kg ton<sup>-1</sup> fish (Fig. 2). It was determined that the effects of the interaction between the "diet protein source×phytase enzyme" factors on the amount of the dissolved phosphours released from the trial groups were insignificant ( $p>0.05$ ); however, the effects of the "diet protein source" and "phytase enzyme" factor levels on the amount of the dissolved phosphours released from the trial groups were determined as significant ( $p<0.05$ ). The highest dissolved phosphorus released from the trial groups was recorded in the D<sub>1</sub>+ group as 10.27 kg ton<sup>-1</sup> fish and the lowest was detected in the D<sub>3</sub>-group as 3.88 kg ton<sup>-1</sup> fish (Fig. 2).

## Discussion

In the present trial, it was determined that the total solid waste released to the receiving environment per ton of fish was 43-510 kg, and that this value was quite high according to the Hasan (2001), Azevedo *et al.* (2011) and Ballester-Moltó *et al.* (2017). We are of the opinion that the reason for this difference is that the study was carried

out in tanks. Results of the present study indicate that there were relative decreases of 11 %, and 24 % in the solid waste discharge amounts of the groups fed with D<sub>2+</sub>, D<sub>3+</sub> phytase added diets, respectively.

It was determined that the total and the particle nitrogen discharge were not affected by the factor levels and it was evaluated that the total nitrogen discharge per ton of fish was 55.61-65.41kg (Table 3). These values were determined to be slightly higher than the findings of Boyd and Queiroz (2001), Azevedo *et al.* (2011), Maar *et al.* (2018), similar to those of Aşır and Pulatsü (2008), and lower than those of Ackefors and Enell (1990), Foy and Rosell (1991), Hasan (2001). We are of the opinion that these differences can originate from many factors such as aquaculture settings, diet structure, and the size of the fish.

The particle nitrogen discharge amount was calculated as 13.99-17.89 kg ton<sup>-1</sup> fish (Table 3). It was determined that the diet was not affected by the protein sources and the phytase enzyme levels. These results are similar to those of Ackefors and Enell (1990), Azevedo *et al.* (2011), and Maar *et al.* (2018).

It was determined that the effects of the protein sources of the diet and the phytase enzyme levels were significant on the dissolved nitrogen discharge amount, and this value was calculated as 43.88-51.33 kg per ton of fish (Table 3). This value is higher than those of Azevedo *et al.* (2011) and Maar *et al.* (2018), and slightly lower than those of Ackefors and Enell (1990). An increase

was detected in the dissolved nitrogen amount released from the groups fed with the diets, which contained phytase enzyme and which had the same protein sources and rates. However, the increase of the herbal protein sources in the diets increased the dissolved nitrogen discharge amount. It can be mentioned that this result is similar to the results of Herath and Satoh (2015), however, it is different from the data of Vielma *et al.* (1998), Vielma *et al.* (2000), Cao *et al.* (2007), Crab *et al.* (2007), Dalsgaard and Pedersen (2011), Liu *et al.* (2009) and Orisasona *et al.* (2017).

The effects of the phytase enzyme levels on particle and dissolved phosphorus discharge amounts were determined as significant. The total phosphorus discharge amount was determined as 11.64-15.79 kg per ton of fish. This value was different from the results of Biswas *et al.* (2007), Cao *et al.* (2007), and Dalsgaard *et al.* (2009), but it was similar to the data of Midlen and Redding (1998), Hasan (2001), Aşır and Pulatsü (2008), David *et al.* (2015). It was observed that the discharge amount was lower compared to the findings of Azevedo *et al.* (2011), and Foy and Rosell (1991); however, it was higher compared to the data of Ackefors and Enell (1990), Hernandez *et al.* (2004), Pulatsü *et al.* (2004), and Maar *et al.* (2018). Phytase addition to the diets increased the total phosphorus discharge.

It was found that the effects of diet protein sources on the phosphorus discharge were insignificant; however, those of phytase enzyme addition were

significant. This value was calculated as 5.42-8.45 kg per ton of fish, and was observed to be similar to the data of Ackefors and Enell (1990), Boyd and Queiroz (2001), while it was lower than the values of Maar *et al.* (2018). Phytase enzyme addition decreased the particle phosphorus discharge amount ( $p<0.05$ ). It can be mentioned that the use of herbal protein sources positively influenced the particle phosphorus discharge. It was calculated that the dissolved phosphorus discharge amount per ton of fish was 3.88-10.27 kg. It was found that the effects of protein sources of the diet and the phytase enzyme levels were significant on this value ( $p<0.05$ ). It was determined that the dissolved phosphorus amount discharge decreased as the herbal protein sources increased in the diet, however, the addition of phytase enzyme increased this discharge amount. This value was similar to the data of Cheng *et al.* (2010); Dalsgaard *et al.* (2009), Azevedo *et al.* (2011), and Maar *et al.* (2018).

In conclusion, it was determined that the dissolved nitrogen amount released from the group fed with D<sub>1+</sub> diet, which contained high proportions of herbal protein and phytase enzyme, was significantly higher compared to the group fed with D<sub>3-</sub> diet, which did not contain phytase enzyme but was low in the proportion of herbal protein ( $p<0.05$ ). Dissolved nitrogen release, which is a bigger problem for the environment, took place at higher levels than the others.

The amounts of hazelnut and soybean meals used in the trial diets in

ascending order, were  $D_1>D_2>D_3$ , respectively. It was observed that there was less total phosphorus discharge ( $p<0.05$ ) in the groups fed with D<sub>1-</sub> and D<sub>3-</sub> diets, which did not contain phytase enzyme.

The dissolved phosphorus discharge released from the groups fed with D<sub>1+</sub>, D<sub>2+</sub> and D<sub>3+</sub> diets, which contained phytase enzyme in ascending order were determined as  $D_{1+}>D_{2+}>D_{3+}$ , respectively; and in the groups fed with the D<sub>1-</sub>, D<sub>2-</sub> and D<sub>3-</sub> diets, which did not contain phytase enzyme, the highest level of dissolved phosphorus release was detected as  $D_{3-}>D_{1-}>D_{2-}$ .

A negative relation was detected between the digestibility rate of the trial diet proteins and discharge amount of the total nitrogen and phosphorus. This negative relation increases as the amounts of soybean meal and hazelnut meal increases in the diet. No significant difference was determined between the digestibility rates of all of the isonitrogenic trial diet proteins containing protein sources and phytase enzymes in different amounts.

Total and dissolved nitrogen discharge amount increased as the soybean and hazelnut meals increased. Moreover, phytase enzyme addition had a similar influence. The effects of the factors on the particle nitrogen discharge amounts were determined to be similar. It was also determined that the groups, which were fed with diets containing phytase enzyme, showed an increase in the amount of the total and dissolved phosphorus discharge, however, the amount of the particle phosphorus discharge decreased

compared to the groups which were fed with diets without phytase enzymes.

In this context, in order to provide the ecological requirements for the development of the fish, optimization of the feed production techniques and the proportions of herbal and animal-origin raw materials in feed formulations are vital for sustainable aquaculture.

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